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During the first year of this award, we established that the α6β1 integrin plays an important role in regulating the growth and survival of breast carcinoma. We developed a dominant negative strategy to 'knock-out' the expression of the α6β1 integrin in a highly metastatic breast carcinoma cell line (MDA-MB-435). After depletion of α6β1 surface expression, these cells are deficient in their growth in the mammary fat pad of athymic mice and can no longer survive as metastases in the lungs and liver. We also established a mechanism for the involvement of the α6β4 integrin in promoting carcinoma invasion. We isolated MDA-MB-435 transfectants that express the α6β4 integrin receptor. These cells have a marked increase in their ability to invade through basement membrane matrices *in vitro*. The mechanism by which this integrin promotes invasion involves a preferential and localized targeting of phosphoinositide-3 OH kinase (PI 3-K) activity. The small GTP-binding protein Rac is downstream of PI 3-K in the cells examined and it is involved in invasion. These findings establish a novel function for PI 3-K signaling and suggest that this α6β4/PI 3-K signaling pathway is a potential target for inhibiting tumor spread.

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Introduction

A major advance in cancer research made during the last several years has been the realization that integrin adhesion receptors play a major role in regulating tumor behavior. Specific tumor-associated functions that are regulated by integrins include adhesion and migration, differentiation, growth and apoptosis, and angiogenesis (1-5). This array of diverse but important functions reflects the fact that integrins are critical for the function and maintenance of the normal epithelial progenitors of all carcinomas. The importance of integrin-mediated interactions in normal epithelial biology has been particularly well-demonstrated for the mammary epithelium (6). Such studies have identified the laminins, a family of extracellular matrix proteins, as key players in determining the function of both normal and transformed mammary epithelia. For these reasons, integrin laminin receptors are prime candidates for investigating the role of cell adhesion events in breast carcinoma (7).

Although several integrin heterodimers can function as receptors for members of the laminin family, the α6 integrins, α6β1 and α6β4, are the predominant laminin receptors in many cell types (7). The possibility that the α6 integrins play an important role in breast carcinoma progression is supported by a study by Imhof and colleagues (8) which examined the expression of α6 in invasive breast carcinomas of 119 women and correlated levels of expression with patient survival. The results demonstrated that high expression levels of the α6 subunit correlated with reduced patient survival time. In fact, α6 integrin expression was superior in predicting reduced survival than other known factors alone including estrogen receptor status. These data implicate a key role for the α6 integrins in deadly breast carcinomas. Data to support the involvement of the α6 integrins in the progression of other carcimomas has also been reported. Expression of the α6 integrins in prostate carcinoma cell lines correlates directly with the invasive potential of these cells in nude mice (9). In addition, expression of the α6β4 integrin has been reported to correlate with the progression of squamous, gastric, and colon carcinomas (10-12). Taken together, these data, in conjunction with the Imhof data, provide a compelling case for the involvement of α6 integrins in carcinoma progression. However, these studies only emphasize the potential importance of the α6 integrins in breast cancer, but they do not provide mechanistic information. Until this is established, the full potential of α6 for diagnosis, or as a target for therapeutic development, will not be known.

The ability of integrin receptors to influence cellular processes such as growth, differentiation, cell motility, and tumor progression is the result of both their adhesive and signaling capabilities (1,13). Many of the signaling pathways that have been characterized for growth factor receptors can be stimulated by integrin receptor engagement. In fact, these two receptor systems may act in concert to elicit the specific signals that are required for proper cell function (14). There is still much to be learned about how integrin receptors control signaling pathways but it is clear that alterations in integrin expression and function during transformation are likely to have multiple consequences on tumor progression. The purpose of the work supported by this grant is to elucidate specific functions of breast carcinoma cells that are influenced by the α6β1 and α6β4 integrins and to elucidate the signaling pathways that control these functions. The work performed during the first year of this award has made significant progress toward attaining this goal.

Body

The α6β1 integrin is required for the growth and survival of MDA-MB-435 breast carcinoma cells *in vivo*. One of the Specific Aims of this grant was to 'Assess the functional significance of the α6β1 integrin in the tumorigenic and metastatic behavior of breast carcinoma cells'. To examine the contribution of the α6β1 integrin to breast carcinoma progression, we developed a dominant-negative technique for 'knocking-out' the expression of α6β1 in MDA-MB-435 cells, a human breast carcinoma cell line that is highly metastatic in athymic mice. A mutant β4 subunit that lacked the entire cytoplasmic domain was expressed at high levels in this cell line. β4 dimerizes only with α6, and not other integrin α subunits, and therefore it selectively depleted α6β1 surface expression by forming the α6β4-ΔCYT heterodimer. We found that elimination of α6β1 expression inhibited the ability of these cells to mediate specific *in vitro* functions associated with metastatic spread such as laminin adhesion and migration (*Shaw, L.M., C. Chao, U.M. Wewer, and A.M. Mercurio. 1996. Function of the integrin α6β1 in metastatic breast carcinoma cells assessed by expression of a dominant negative receptor. Cancer Res., 56:959-963.*). Subclones of these transfectants were isolated by FACS sorting. Mock (transfected with the vector alone) and β4-ΔCYT transfectant subclones were chosen that expressed similar levels of all integrin subunits. These subclones also adhered to other matrix proteins such as fibronectin, vitronectin, and collagen I to the same extent.

In collaboration with Ulla Wewer in Copenhagen, Denmark, we examined the *in vivo* behavior of MDA-MB-435 breast carcinoma cells that lack α6β1 expression. Mock and α6β4-ΔCYT transfectant subclones were injected into the mammary fat pad of female athymic mice. After several weeks, the mice were sacrificed and the extent of tumor growth, invasion, and metastasis was analyzed. Distant metastasis was examined by sectioning the lungs and livers and counting metastatic foci.

Cell Type	# Mice	Weight (g) (primary tumor)	# Mice with Lung Metastases	Mitotic Index	Apoptotic Index
Mock	**53**	**3.9 (±2.0)**	**45 (85%)**	**1.05±0.29**	**0.48±0.26**
α6β4-ΔCYT	**75**	**0.6 (±0.5)**	**2 (3%)**	**0.48±0.16**	**1.41±0.31**

Table 1: Analysis of α6β1 function in breast carcinoma behavior *in vivo*. MDA-MB-435 cells that either expressed α6β1 (Mock) or lacked expression of α6β1 (α6β4-ΔCYT) were injected into the mammary fat pad of female nu/nu mice. After several weeks of growth the mice were sacrificed, the primary tumors removed and weighed, and distant metastasis to the lungs was quantified. The mitotic and apoptotic indices were derived by counting the number of mitotic and apoptotic cells respectively in approximately 1000 viable tumor cells. Apoptotic cells were identified by morphological criteria and confirmed by Apoptag staining.

Although metastatic foci were not present in the lungs of the β4-ΔCYT transfectant mice, small foci of apoptotic cells were observed. These data suggest that the β4-ΔCYT transfectants were able to metastasize to the lungs but were not capable of surviving in this environment. In addition, the mock and β4-ΔCYT transfectants invaded through the abdominal wall into the peritoneal cavity in a similar percentage of mice. Taken together, these data indicate that the loss of α6β1 expression does not compromise the invasive potential of these breast carcinoma cells, a hypothesis that was predicted before these experiments were carried out. Rather, the results from the *in vivo* studies demonstrate that the α6β1 integrin functions in the growth and survival of breast carcinomas. (See appendix: *Wewer, U.M, L.M. Shaw, R. Albrechtsen, and A.M. Mercurio. 1997. The integrin α6β1 promotes the survival of metastatic human breast carcinoma cells in mice. Amer. J. Path. [in press]*). Work is presently focused on understanding the signaling pathways that are regulated by the α6β1 integrin that contribute to growth and survival.

Expression of the β4 integrin subunit in MDA-MB-435 cells. Expression of the β4 integrin subunit correlates with the progression of several carcinomas including colon, squamous, and gastric (10-12). The correlation of β4 expression with breast carcinoma progression is not well established, although some reports have suggested this possibility. For example, in one study, the β4 subunit was shown to be more highly expressed in metastases than in the corresponding primary tumors (16). To study the signaling functions of the α6β4 receptor and their contribution to tumor progression more rigorously, we established MDA-MB-435 breast carcinoma transfectants that express the intact β4 subunit (Specific Aim #4: Assess the consequence of restoring α6β4 function in β4-negative breast carcinoma cells). Subclones have been isolated that express high levels of the α6β4 receptor on the cell surface (Figure 1).

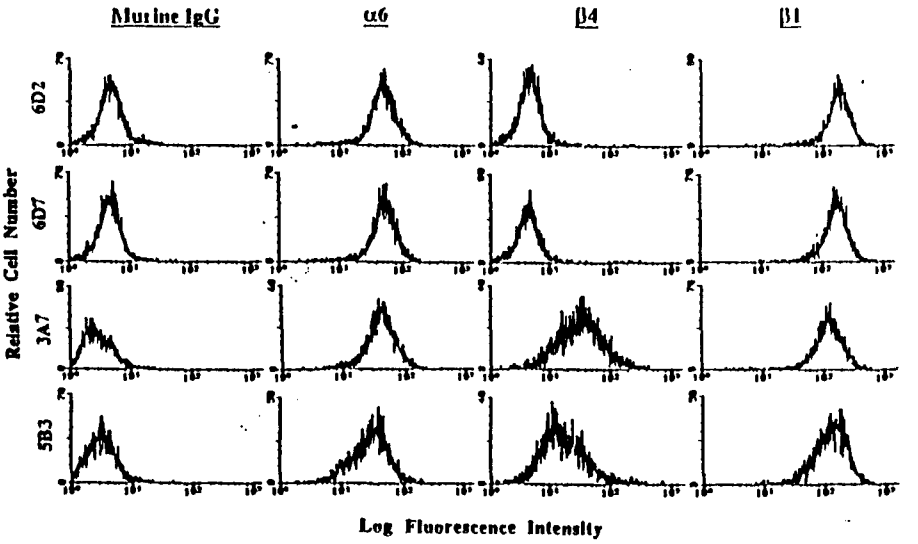

Figure 1: Expression of the β4 integrin subunit in MDA-MB-435 breast carcinoma cells. MDA-MB-435 breast carcinoma cells were transfected with the human β4 cDNA (α6β4) or the expression vector alone (Mock). β4-specific antibodies were used to sort the population of cells that express the β4 subunit on the cell surface and these cells were then subcloned. Shown are two representative Mock (6D2 and 6D7) and α6β4 (3A7 and 5B3) subclones.

Expression of the α6β4 integrin increases the invasiveness of MDA-MB-435 cells MDA-MB-435 breast carcinoma transfectants that express the intact β4 subunit were examined for their ability to invade Matrigel in a standard chemoinvasion assay. The rate of invasion of the β4 transfectants was approximately 3-4 fold greater than that of the mock transfectants in a 4 hour assay. The β4-ΔCYT transfectants invaded at a slightly slower rate than that of the mock transfectants (Fig. 2A) indicating that the β4 cytoplasmic domain is essential for stimulating invasion. To examine the contribution of integrin receptors to the invasion of MDA-MB-435 cells, Matrigel chemoinvasion assays were performed in the presence of integrin subunit specific antibodies. A β1-specific antibody (mAb 13) inhibited invasion of the mock and β4-transfectants (Fig. 2B). An α6-specific mAb (2B7) inhibited invasion of the mock transfectants by approximately 60% (Fig. 2B), in agreement with our previous result that these cells use α6β1 as a major laminin receptor. However, the same antibody increased the rate of invasion of the β4 transfectants by approximately 30% (Fig. 2B). The stimulation of invasion observed for the α6 antibody in the MDA-MB-435/β4 transfectants suggests that α6β4 is not required for the adhesive functions involved in invasion but rather acts as a signaling receptor whose function can be enhanced by antibody binding.

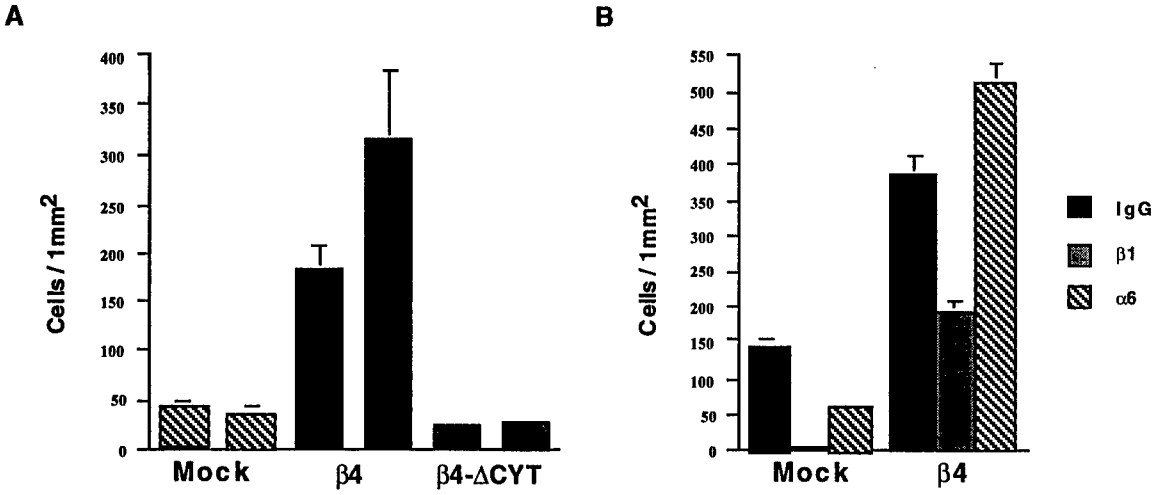

Figure 2: Invasion of the MDA-MB-435 transfectants. A) MDA-MB-435 transfectants were assayed for their ability to invade Matrigel. Matrigel was diluted in cold distilled water, added to the upper well of Transwell chambers, and dried in a sterile hood. The Matrigel was reconstituted with medium and the transfectants were added to each well. Conditioned NIH-3T3 medium was added to the bottom wells of the chambers. B) MDA-MB-435 transfectants were assayed for their ability to invade Matrigel in the presence of monoclonal antibodies specific for the indicated integrin subunits. Cells were preincubated for 30 minutes in the presence of antibodies before addition to the Matrigel-coated wells. After 4 hrs, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated. Mock, MDA-MB-435 cells transfected with vector alone; β4, MDA-MB-435 cells transfected with the full length β4 subunit; β4- ΔCYT, MDA-MB-435 cells transfected with the β4 subunit lacking the cytoplasmic domain; IgG; non-specific antibody; β1, mAb 13; α6, 2B7.

Invasion of MDA-MB-435 cells is dependent on PI 3-K. As a prelude to identifying the signaling mechanism by which the α6β4 integrin stimulates invasion, we first assessed the effects of the MAPK kinase inhibitor PD98059 on MDA-MB-435 invasion (20). We selected this pathway because it has been reported that MAPK contributes to cell motility by phosphorylating myosin light chain kinase (21). As shown in Fig. 3, pre-treatment of these cells with PD98059 (25 uM) resulted in only a modest inhibition (20%) of invasion. We next targeted PI 3-K because of its central involvement in multiple signaling pathways (22). The PI 3-K inhibitor wortmannin inhibited invasion of both the mock and β4 transfectants by 60-80% (23; Fig. 3). These results suggest that PI 3-K, but not MAPK, is necessary for the invasion of the MDA-MB-435 cells.

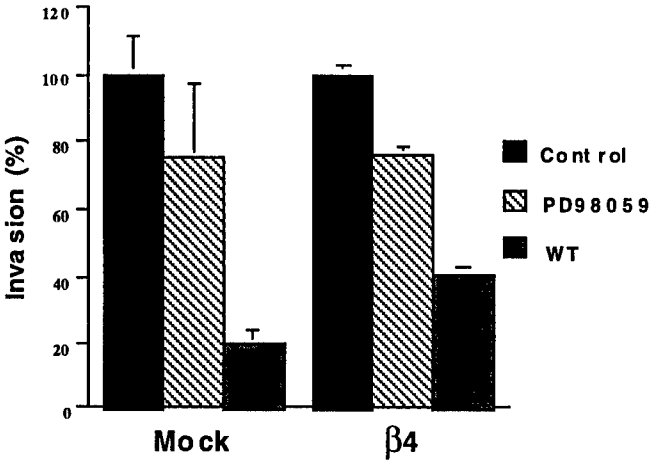

Figure 3: Analysis of MAPK and PI 3-K involvement in MDA-MB-435 invasion. MDA-MB-435 transfectants were assayed for their ability to invade Matrigel in the presence of the MEK inhibitor PD98059 (25uM) or the PI 3-K inhibitor wortmannin (WT;100uM). Cells were preincubated for 10 minutes in the presence of the inhibitors before addition to the Matrigel-coated wells. After 4 hrs, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated. The data shown are the mean values (±SD) of one experiment done in duplicate. Mock, MDA-MB-435 cells transfected with vector alone; β4, MDA-MB-435 cells transfected with the full length β4 subunit.

Activation of PI 3-K by the α6β4 integrin To determine if the α6β4 integrin can stimulate PI 3-K activity, *in vitro* kinase assays were performed using the mock, β4, and β4-ΔCYT transfectants of MDA-MB-435 cells. After ligation of the α6 integrins with 2B7, extracts were immunoprecipitated with a phosphotyrosine-specific antibody to capture the activated population of PI 3-K and these immunoprecipitates were assayed for their ability to phosphorylate crude brain phosphoinositides. As shown in Fig. 4A, an increase in PI 3-K activity, indicated by the appearance of PtdIns-3,4,5-P_3, was observed upon clustering the α6β1 integrin in the mock transfectants and the α6β4 integrin in the β4 transfectants. More importantly, PI 3-K activity stimulated by clustering the α6β4 integrin was markedly greater than that observed after clustering the α6β1 receptor. This enhanced stimulation of PI 3-K was also seen using a β4-specific mAb to ligate the α6β4 integrin in the β4 transfectants (Fig. 4A). PI 3-K activity was also higher in the β4 transfectants than in the mock transfectants after adhesion to laminin-1 (Fig. 4A). This observation suggests that even though α6β4 is not used as the major adhesion receptor in these cells, interactions with laminin through this receptor can stimulate PI 3-K activity. PI 3-K activity was not increased upon ligation of the α6β4-ΔCYT receptor and little activity was evident when the transfectants were maintained in suspension (Fig. 4).

Our data suggested that the ability of the α6β4 integrin to activate PI 3-K may be quantitatively greater than that of β1 integrins in MDA-MB-435 cells. This possibility was examined by comparing PI 3-K activation in the β4 transfectants in response to antibody-ligation of either β1 integrins or the α6β4-integrin. As shown in Fig. 4B, ligation of the α6β4 integrin with β4-specific antibodies stimulated PI 3-K activity approximately two fold greater than β1 integrin ligation demonstrating that PI 3-K is activated preferentially by the α6β4 integrin. The differences between the abilities of the α6β4 and β1 integrins to activate PI 3-K are most likely even greater than what was observed given the two-three fold higher level of expression of β1 than β4 on the cell surface (Fig. 1).

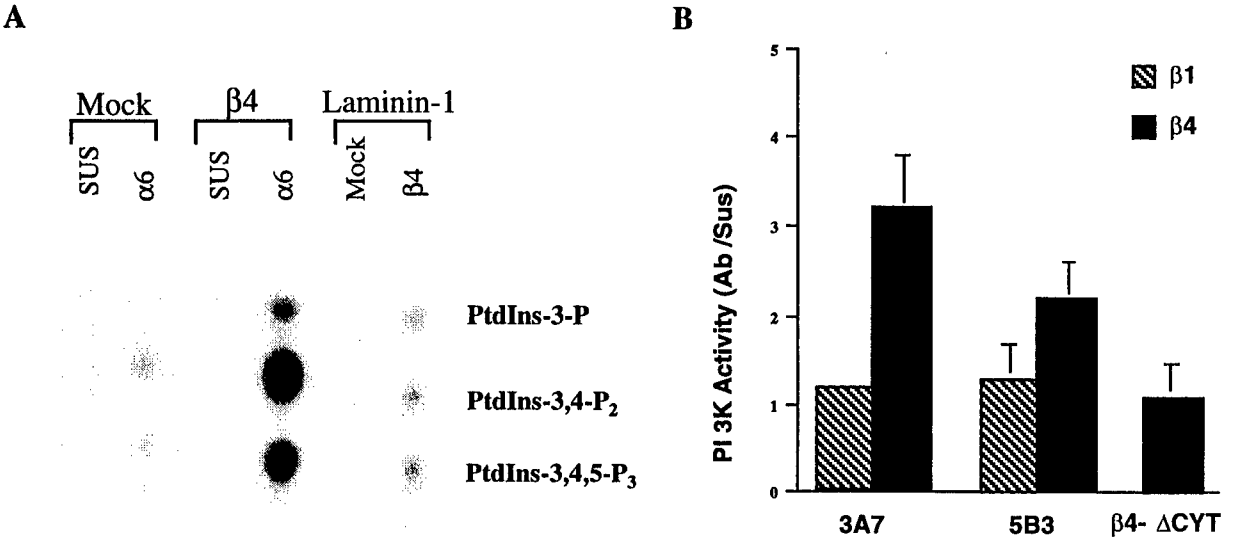

Figure 4: Analysis of PI 3-K activity in the MDA-MB-435 transfectants. MDA-MB-435 transfectants were maintained in suspension or incubated with α6-, β1-, or β4-specific antibodies and allowed to adhere to anti-mouse IgG coated plates or laminin-1 coated plates for 30 minutes. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with the anti-phosphotyrosine mAb 4G10 and Protein A sepharose for 3hrs. After washing, the beads were resuspended in kinase buffer and incubated for 10 minutes at room temperature. The phosphorylated lipids were resolved by thin layer chromatography. A) The D3-phosphoinositides are indicated by arrows. B) The amount of radiolabeled PtdIns-3,4,5-P_3 was determined for each condition by densitometry. The integrin activated levels of PtdIns-3,4,5-P_3 were compared to the level observed for the cells that were maintained in suspension. The value from this ratio was determined to be the relative PI 3-K activity stimulated by each integrin subunit. Mock, MDA-MB-435 cells transfected with vector alone; β4, MDA-MB-435 cells transfected with the full length β4 subunit; SUS, cells maintained in suspension; α6, cells clustered with the α6-specific antibody.

Constitutively active PI-3K stimulates invasion of MDA-MB-435 cells The hypothesis that the α6β4 integrin promotes invasion of MDA-MB-435 cells by enhancing the activity of PI 3-K implies that: 1) expression of a constitutively active form of PI 3-K in the parental cells should increase their invasion in the absence of α6β4 expression and 2) expression of a dominant negative PI 3-K subunit in the β4 transfectants should decrease their invasion. To validate these predictions, a constitutively active, membrane-targeted PI 3-K (Myr-p110-Myc) was expressed transiently in MDA-MB-435 cells and the ability of these cells to invade Matrigel was compared to cells transfected with an empty vector. As shown in Fig. 5A, constitutively active PI 3-K increased invasion two-fold and this invasion was inhibited by wortmannin. Overexpression of the wild type p85 regulatory subunit has been demonstrated to function in a dominant negative manner and inhibit endogenous PI 3-K activation (24). Transient expression of a myc-tagged p85 subunit inhibited the invasion of the MDA-MB-435/β4 transfectants by 50% (Fig. 5B). The data obtained with the wild-type p85 subunit substantiate the wortmannin data shown in Fig.3.

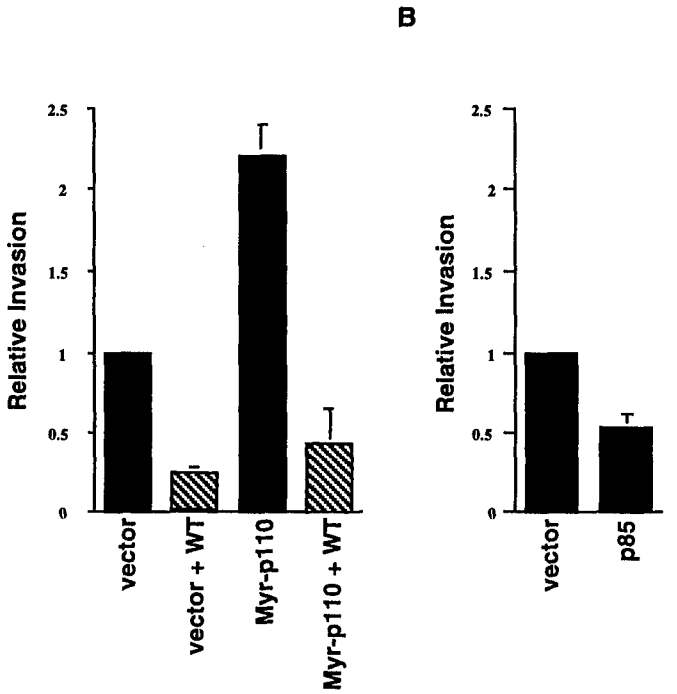

Figure 5: Analysis of PI 3-K involvement in invasion of MDA-MB-435 cells by transient transfections. A) MDA-MB-435 cells were transiently transfected with a Myc-tagged constitutively active form of the PI 3-K p110 catalytic subunit, Myr-p110, and assayed for their ability to invade Matrigel in the absence or presence of wortmannin (WT; 100nM). B) MDA-MB-435/b4 transfectants were transiently transfected with a wild-type PI 3-K p85 regulatory subunit, p85, and assayed for their ability to invade Matrigel. After 5 hrs, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed and stained. Invasion was quantitated by counting the cells that stained positively for β-galactosidase expression. Relative invasion was determined by comparing the amount of invasion obtained for the experimental transfections to that observed for the cells transfected with the vector alone, which was given the value of 1.

The Akt/PKB kinase, a downstream effector of PI-3K, is not required for invasion The Akt/PKB serine/threonine kinase (Akt) is activated downstream of PI 3-K and, for this reason, could play an important role in invasion (25). This possibility was supported by our finding that ligation of the α6β4 integrin in the MDA-MB-435 transfectants can activate Akt. Based on these observations, we examined the ability of MDA-MB-435 cells that expressed a constitutively active form of Akt (Myr-Akt) to invade Matrigel. Surprisingly, this constitutively active form of Akt actually decreased the rate of invasion in comparison to the control cells even though it was expressed at relatively high levels (Fig. 6A). Most likely, the exogenously expressed Akt sequestered a significant fraction of D3 phosphoinositides and precluded the use of these lipids in those signaling pathways downstream of PI 3-K that are required for invasion. Based on these results, we conclude that Akt is not required for MDA-MB-435 invasion.

The small G-protein Rac is required for MDA-MB-435 invasion The small G-protein Rac is a downstream effector of PI 3-K that is involved in the actin rearrangements that result in the formation of membrane ruffles and lamellae (26). The ability of cells to form lamellae is strongly

correlated with their motility and therefore influences their invasive potential. To examine the role of Rac in PI 3-K dependent invasion, a dominant negative mutant of Rac, N17Rac, was transiently expressed along with the Myr-p110-Myc construct in the MDA-MB-435 cells. As shown in Fig. 6A, co-expression of the N17Rac inhibited the increased invasion that was observed when the constitutively active p110 subunit of PI 3-K was expressed alone. In addition to this experiment, the N17Rac construct was expressed transiently in the MDA-MB-435/β4 transfectants and a 50% reduction in invasion was observed (Fig. 6B). Taken together, these results demonstrate that Rac is an essential downstream mediator of the α6β4/PI 3-K signaling pathway involved in invasion. Expression of a constitutively active mutant of Rac, V12Rac, did not significantly increase the invasion of the MDA-MB-435 cells suggesting that other PI-3K downstream effectors, in addition to Rac, are important for invasion in these cells. (data not shown).

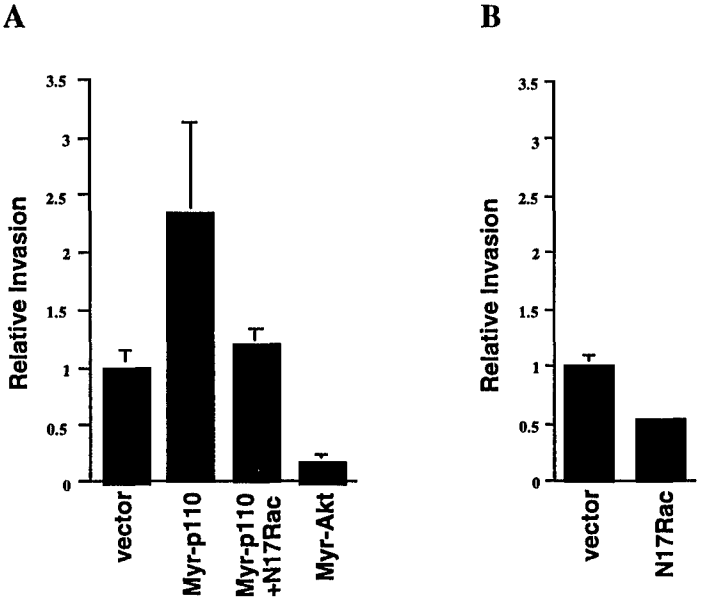

Figure 6: Analysis of Rac and Akt involvement in PI 3-K-dependent invasion of MDA-MB-435 cells. A) MDA-MB-435 cells were transiently transfected with either the vector alone, Myr-p110, Myr-p110 and a dominant negative mutant of Rac, GST-N17Rac, or a constitutively active form of Akt, Myr-Akt, and assayed for their ability to invade Matrigel. B) MDA-MB-435/β4 transfectants were transiently transfected with vector alone or N17Rac and assayed for their ability to invade Matrigel. After 5 hrs, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed and stained. Invasion was quantitated by counting the cells that stained positive for β-galactosidase expression. Relative invasion was determined by comparing the amount of invasion obtained for the experimental transfections to that observed for the cells transfected with the vector alone, which was given the value of 1.

Conclusions

In order to take full advantage of the α6 subunit as a marker for predicting the prognosis of breast cancer, it is necessary to understand mechanistically how the α6β1 and α6β4 integrins promote aggressive tumor behavior. Until this is established, the full potential of α6 for diagnosis, or as a target for therapeutic development, will not be known. The goal of this grant is to examine the role of the α6 integrins in breast carcinoma progression. Extensive progress has been made during this first year on two of the Specific Aims of the grant. The work to date from this award has demonstrated that the α6 integrins have specific roles in breast carcinoma progression that involve distinct signaling functions of α6β1 and α6β4.

Elimination of α6β1 expression from a highly metastatic breast carcinoma cell line inhibited the ability of these cells to metastasize in athymic mice. However, this was not due to an inability to invade as was predicted from *in vitro* experiments that showed a decreased ability to adhere and migrate on laminin substrates. Rather, the decreased metastasis appears to be due to the inability of these cells to survive in distant organs. These cells are also inhibited in their primary tumor growth because of a decreased proliferative rate and an increased apoptotic rate. Therefore, the conclusion can be drawn that the α6β1 integrin contributes to breast carcinoma progression by controling signaling pathways that are involved in cell cycle progression and/or apoptotic regulation. The work in the future years of this grant will focus on identifying specific signaling pathways that are activated by the α6β1 integrin that may contribute to these functions.

The α6β4 integrin regulates signaling pathways that influence carcinoma invasion. Expression of the β4 integrin subunit in a highly metastatic breast carcinoma cell line markedly increased the *in vitro* invasive potential of these cells. The mechanism by which this integrin promotes invasion involves a preferential and localized targeting of phosphoinositide-3 OH kinase (PI 3-K) activity. Exogenous expression of α6β4 increased carcinoma invasion in a PI 3-K-dependent manner and transient expression of a constitutively active PI 3-K increased invasion in the absence of α6β4. Ligation of α6β4 stimulated significantly more PI 3-K activity than ligation of either α6β1 or other β1 integrins establishing specificity among integrins for PI 3-K activation. An essential role for PI 3-K in invasion constitutes a novel function for this kinase and implies that downstream effectors of PI 3-K are critical for the invasive process. We provide evidence in fact that the small GTP-binding protein Rac is downstream of PI 3-K in the cells examined and that it is involved in invasion. In contrast, the serine/threonine kinase Akt does not contribute to the invasive process even though it is regulated by PI 3-K and is activated by the α6β4 integrin. Collectively, our findings provide a mechanism for the involvement of α6β4 in promoting carcinoma invasion and invoke a novel function for PI 3-K signaling. These data support the previous findings that the expression of α6β4 is associated with invasive carcinoma. A fascinating problem is raised by these observations because carcinoma progression involves both tumor cell invasion and survival. The recent demonstration that Akt is required for the survival of several cell types (17,18) coupled with our finding that PI-3K is required for invasion suggests that two essential functions of progression may require the products of PI 3-K and that the balance between the use of these pathways may impact tumor cell invasion or survival. These findings are particularly important because they suggest that this α6β4/PI 3-K signaling pathway is a potential target for inhibiting tumor spread.

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Short Communication

The Integrin α6β1 Promotes the Survival of Metastatic Human Breast Carcinoma Cells in Mice

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The role of the integrin α6β1 in breast carcinoma progression was studied by targeted elimination of this integrin in MDA-MB-435 cells, a human breast carcinoma cell line that is highly metastatic in athymic mice. The strategy used is based on the finding that expression of a cytoplasmic domain deletion mutant of the β4-integrin subunit (β4-ΔCYT) in MDA-MB-435 cells eliminates formation of the α6β1 heterodimer. MDA-MB-435 cells that lacked α6β1 expression (β4-ΔCYT transfectants) formed tumors in athymic mice that were suppressed in their growth and that exhibited a significant increase in apoptosis in comparison to the control tumors. Unlike the control MDA-MB-435 cells, the β4-ΔCYT transfectants were unable to establish metastatic foci in the lungs. Also, the control transfectants grew substantially better than the β4-ΔCYT transfectants in the liver after intrahepatic injection because of extensive apoptosis in the β4-ΔCYT transfectants. These data suggest that a major function of the α6β1 integrin in breast carcinoma is to facilitate tumorigenesis and promote tumor cell survival in distant organs. ***(Am J Pathol 1997, 151:000–000)***

Cell adhesion receptors including integrins are likely to play critical roles in the metastatic progression of breast and other carcinomas.[1–4] The contribution of such receptors to metastasis probably involves their adhesive functions as well as their ability to stimulate signaling pathways that influence tumor behavior. It is important, therefore, not only to establish the involvement of specific integrins in progression but also to understand how these integrins actually contribute to the metastatic process. In this direction, we have been interested in assessing the involvement of the α6β1 integrin in breast carcinoma progression. This integrin functions as an adhesion receptor for the laminin family of basement membrane proteins[5] as well as for at least one member of the disintegrin family of surface receptors.[6] The expression of this integrin in normal breast tissue, benign lesions, and neoplastic disease has been studied by several groups.[7–13] Although no consensus has emerged from these studies with respect to disease stage and α6β1 expression, one study of particular interest observed that expression of α6 integrins in women with breast cancer correlates with reduced survival times.[13] In fact, α6-integrin expression was found to be superior in predicting reduced survival than other known factors alone, including the estrogen receptor.[13]

To examine the hypothesis that the α6β1 integrin functions in breast carcinoma progression more rigorously, we developed a technique for eliminating its expression in MDA-MB-435 cells,[14] a human breast carcinoma cell line that is highly metastatic in athymic mice.[15] MDA-MB-435 cells use the α6β1 integrin as their only laminin-1 receptor, and they do not express the α6β4 integrin laminin receptor.[14] The strategy used is based on our observation that expression of a cytoplasmic domain deletion mutant of the β4-integrin subunit in MDA-MB-435 cells eliminates formation of the α6β1 heterodimer. Targeted removal of the α6β1 integrin in these breast carcinoma cells inhibited their ability to mediate specific *in vitro* functions associated with tumor spread such as adhesion and migration through laminin-containing matrices.[14] In the present study, we assessed the involvement of the α6β1 integrin in spontaneous metastasis using these MDA-MB-435 transfectants. MDA-MB-435 cells that lacked α6β1 expression formed tumors in athy-

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mic mice that were suppressed in their growth and exhibited a significant increase in apoptosis in comparison with the control tumors. Unlike the control MDA-MB-435 cells, the $\alpha 6\beta 1$-deficient cells were unable to establish metastatic foci in the lungs possibly because they became apoptotic after extravasation into this organ. The data presented suggest that a major function of the $\alpha 6\beta 1$ integrin in breast carcinoma is to facilitate tumorigenesis and promote tumor cell survival in distant organs.

Materials and Methods

Analysis of MDA-MB-435 Transfectants

Subclones of MDA-MB-435 cells that had been transfected with either the pcDNA3 vector alone (control transfectants) or with the $\beta 4$-integrin cytoplasmic domain deletion mutant in this vector ($\beta 4$-ΔCYT transfectants) were generated as described previously.[14] The surface expression of integrin subunits on the control and $\beta 4$-ΔCYT transfectants was assessed by flow cytometry. For this purpose, aliquots of cells (5×10^5) were incubated for 45 to 60 minutes at room temperature with RPMI 1640 medium containing 25 mmol/L Hepes (RPMI-H) and 0.2% bovine serum albumin (RH/BSA) and the following integrin-specific Abs: HP2B6 ($\alpha 1$, Immunotech); IIE10 ($\alpha 2$, provided by Martin Hemler); IVA5 ($\alpha 3$, provided by Martin Hemler); SAM1 ($\alpha 5$, Immunotech); 2B7 ($\alpha 6$, prepared in our laboratory); AMF7 (αv, Immunotech); mAb 13 ($\beta 1$, provided by Stephen Akiyama); A9 ($\beta 4$, provided by Thomas Carey); and mouse IgG (Sigma). The cells were washed two times with RH/BSA and then incubated with goat $F(ab')_2$ anti-mouse IgG coupled to fluorescein (Tago) for 45 to 60 minutes at room temperature. After washing two times with RH/BSA, the cells were resuspended in the same buffer and analyzed using a FACScan (Becton Dickinson).

In Vivo *Assays*

For injection, the cells were harvested, equilibrated in complete growth medium for 1 hour in suspension at 37°C, rinsed in phosphate-buffered saline, and resuspended at a concentration of 10^8 cells/ml. Female *nu/nu* NMRI mice or META mice (6 to 8 weeks old) (Bomholtgard Breeding and Research Center, Ry, Denmark) were injected with the cells (10^7 per inoculum) into the mammary fat pad region or subcutaneously using a 26-gauge needle. For serial transplantation, small pieces of viable tumor tissue were inoculated subcutaneously in the flank of the thoracic region of the NMRI mice. The mice were maintained in specific pathogen free conditions in sterile laminar flow benches and fed sterile water and food *ad libitum*. Mice were killed by cervical dislocation before the overgrowth of primary tumors or peritoneal tumor growth with ascites fluid, or after 8 to 9 weeks of observation. Upon autopsy, primary tumors were dissected, and the tumor weight was determined. The abdominal cavity was inspected for the presence of macroscopic tumor growth, and tumor tissue and liver specimens were removed. Similarly, the thoracic cavity was inspected, and the lungs and mediastinum were removed. Tissue specimens were fixed in formalin before staining with the ApopTag reagent (Oncor) or in ethanol/acetic acid and processed for H&E histological examination using standard techniques. Tissue specimens were also frozen in liquid nitrogen and stored at −70°C until use for immunohistochemistry with an anti-human p53 protein-specific mAb (Dako, Denmark).

In Situ *Growth and Apoptosis Assays*

The mitotic index (MI) was determined by calculating the percentage of definite mitotic figures in approximately 1000 viable cells within each tumor, and the apoptotic index (AI) was determined by calculating the percentage of apoptotic cells contained within 2000 viable cells within each tumor. Apoptotic cells were identified at the light microscope level by the presence of hyperchromatic compact or fragmented nuclei in single cells within areas of viable tumor cells that were devoid of neutrophils and distant from areas of necrosis. Cells identified by these features also stained positively with the ApopTag reagent in contrast to surrounding viable cells. Intestinal epithelium, which contains apoptotic cells in the villi, served as an internal positive control for these analyses.

Liver Colonization Assay

The liver colonization assay used is based on the procedure described in a study by Kuo et al.[16] Briefly, mice were anesthetized and an incision was made through the upper abdominal wall and peritoneum. The liver was exposed and injected with either cultured tumor cells (2.5×10^6/50 μl) or phosphate-buffered saline for the sham-operated animals using a 26-gauge needle. Mice were killed 4, 11, and 28 days after the injection and autopsied. Histological examination and *in situ* growth and apoptosis assays were performed as described above.

Results

Characterization of the MDA-MB-435 Transfectants

Subclones of MDA-MB-435 cells that had been transfected with either the pcDNA3 vector alone (control transfectants) or with the $\beta 4$-integrin cytoplasmic domain deletion mutant in this vector ($\beta 4$-ΔCYT transfectants) were used in these studies. The control transfectant subclones expressed equivalent levels of $\alpha 6$, and the $\beta 4$-ΔCYT transfectant subclones expressed equivalent levels of both $\alpha 6$ and $\beta 4$. Other integrin subunits ($\alpha 1$, $\alpha 2$, $\alpha 5$, αv, and $\beta 1$) were expressed at equivalent levels although the $\alpha 3$ subunit was expressed at variable levels on both the mock and $\beta 4$-ΔCYT transfectant subclones (Table 1). However, this variability did not correlate with tumorigenesis or metastasis. All of the $\beta 4$-ΔCYT subclones adhered

Table 1. Surface Expression of Integrin Subunits in Mock and β4-ΔCYT Transfectants of MDA-MB-435 Cells

Transfectants	IgG	α1	α2	α3	α5	α6	αv	β1	β4
Mock	4.9	4.4	36.7	29.9	24.5	29.6	20.4	62.5	4.9
β4-ΔCYT	4.9	4.3	40.5	47.3	35.6	22.8	29.8	68.1	23.2

Representative subclones of the mock (B1) and β4-ΔCYT (1E10) transfectants were analyzed by flow cytometry using mAbs specific for the indicated integrin subunits (see Materials and Methods). The data shown represent the mean fluorescence intensity for each integrin-specific mAb as well as for a nonspecific IgG control.

to collagen I, fibronectin, and vitronectin to the same extent as the control transfectants (data not shown). The only observed difference was that the β4-ΔCYT subclones did not adhere well to laminin-1.[14]

The Integrin α6β1 Promotes the Tumorigenic Properties of MDA-MB-435 Cells

Subclones of the control and β4-ΔCYT transfectants were injected into the mammary fat pads in the abdominal region of *nu/nu* NMRI mice. All subclones of the control transfectants grew rapidly and formed large primary tumors (mean tumor weight 2.0 ± 0.13 g) within 7 to 8 weeks (Table 2). Although the β4-ΔCYT subclones were capable of primary tumor growth, these tumors were significantly smaller (mean tumor weight 0.3 ± 0.03 g) than those observed for the control subclones (Table 2). Insight into the size difference between these two groups of primary tumors was obtained by calculating their mitotic and apoptotic indices. Tumors formed by the control transfectants had a higher MI/AI ratio (2.2) than did the β4-ΔCYT tumors (0.34) (Table 3). These data indicate that the β4-ΔCYT tumors were suppressed in their growth and exhibited a significant increase in apoptosis in comparison with the control tumors.

The differences in tumor weight observed for the control and β4-ΔCYT transfectants is not dependent on the environment of the mammary fat pad because such differences were also observed after subcutaneous injection. Specifically, the mean tumor weight observed for the control transfectants (n = 20) was 1.8 ± 0.27 g and for the β4-ΔCYT transfectants (n = 20) was 0.7 ± 0.11 g at 8 weeks after subcutaneous injection. These data were reinforced by serial subcutaneous transplantation of tumor tissue. The mean tumor weight for tumors that grew from transplantation of control transfectant tumors (n = 19) was 2.9 ± 0.35 g and the mean tumor weight for tumors that grew from the β4-ΔCYT transfectant tumors (n = 15) was 0.2 ± 0.03 g.

Growth differences between the control and β4-ΔCYT transfectants were not apparent *in vitro*. Specifically, the mean doubling time (±SEM) for the control transfectant subclones grown on tissue culture plastic in the presence of 5% fetal calf serum was 37.5 ± 2.0 hours. The β4-ΔCYT subclones exhibited a mean doubling time of 35.6 ± 1.3 hours. Although serum deprivation increased these doubling times, the control and β4-ΔCYT subclones did not differ in their ability to survive and proliferate under this condition. Also, there was no evidence of growth suppression of the β4-ΔCYT transfectants when the subclones were grown in three-dimensional cultures

Table 2. Effect of Targeted Elimination of the α6β1 Integrin on the Growth, Peritoneal Spread, and Lung Metastasis of MDA-MB-435 Breast Carcinoma Cells in nu/nu *NMRI Mice*

MDA-MB-435 Transfectants	Number Mice	Time weeks	Tumor weight (g)	Lung Metastasis	
				No mice	%
Control, pcDNA3					
B1, G2 with peritoneal spread	19	6	1.4 ± 0.22	17	90
B1, G2 without peritoneal spread	34	7.3	2.4 ± 0.13	28	82
β4-ΔCYT					
1A2, 1E10, 3C12, 3E3 with peritoneal spread	25	7.8	0.4 ± 0.08	2	8
1A2, 1E10, 3C12, 3E3 without peritoneal spread	50	8.5	0.3 ± 0.03	0	0
Total, Control	53	6.8	2.0 ± 0.13	45	85
Total, β4-ΔCYT	75	8.2	0.3 ± 0.03	2	3

Tumors derived from the β4-ΔCYT transfectants (α6β1 negative) were significantly smaller than tumors derived from the control transfectants. Tumor weight in grams (g) is presented as the mean value (means ± SE). $P < 0.0001$ using the Mann-Whitney test. The same number of mice injected with the control and β4-ΔCYT transfectants exhibited peritoneal spread (36 and 33% of the mice, respectively). This difference is not statistically significant by the chi-square test ($P = 0.54$). The ability of the β4-ΔCYT transfectants to form lung metastases was dramatically reduced (3% of total mice injected) compared with the control transfectants (85% of total mice injected). This difference is highly statistically significant using the chi-square test ($P < 0.000001$). In all experiments, several subclones of each transfectant were tested as indicated and no statistically significant differences among them were observed.

Table 3. In Situ *Growth Analysis of Primary Tumors in MDA-MB-435 Breast Carcinoma Cells in NMRI Mice*

MDA-MB-435 Transfectants	Number of Mice	MI (%) Mitosis	AI (%) Apoptosis	MI/AI Ratio
pcDNA3	10	1.05 ± 0.05	0.48 ± 0.10	2.2
β4-ΔCYT	10	0.48 ± 0.11	1.14 + 0.10	0.34

The mitotic index (MI) and apoptotic index (AI) obtained from the primary tumors formed by the control and β4-ΔCYT transfectants. The MI of the control tumors is significantly higher than that of the β4-ΔCYT tumors ($P < 0.01$ using the Mann-Whitney test). In contrast, the AI of the β4-ΔCYT tumors is significantly higher than that of the control tumors ($P < 0.001$ using the Mann-Whitney test). The MI/AI ratio derived from the control tumors (2.2) is significantly higher than that derived from the β4-ΔCYT tumors (0.34) ($P < 0.002$ using the Mann-Whitney test). The data are presented as mean index (%) and standard error of the mean (Means ± SE).

of Matrigel in either 10% serum or in the absence of serum (data not shown).

Loss of α6β1 expression did not affect the ability of MDA-MB-435 cells to invade from the mammary fat pads in the abdominal region into the peritoneal cavity (Table 2; Figure 1A). Approximately one-third of mice injected in the mammary fat pad with either the control transfectant subclones or β4-ΔCYT transfectant subclones exhibited extensive tumor growth in the peritoneal cavity, although the control transfectants grew better than the β4-ΔCYT transfectants based on visual observation. Both populations of transfectants grew as single cells and clusters in the peritoneal cavity. Tumor nodules were dispersed throughout the peritoneal cavity. In particular, they were seen in omental tissue and surrounding the gastric sac, intestines, spleen, pancreas, and hepatic portal tract. Invasion of tumor cells into the pancreas and blood vessels was also evident (Figure 1B). The β4-ΔCYT transfectants that grew in the peritoneal cavity were not revertants because they stained positively with β4 integrin-specific mAbs (data not shown).

The Integrin α6β1 is Linked to the Survival of Metastatic MDA-MB-435 Cells

The most dramatic effect observed was that expression of α6β1 appears to be essential for the establishment of lung metastases by MDA-MB-435 cells (Table 2; Figure 2). Of the 53 NMRI mice injected with the mock transfectant subclones, 45 developed foci of tumor cells in their lungs. The formation of these lung metastases was not dependent upon peritoneal tumor growth because 82% of the mice that did not exhibit such growth developed these metastases. In marked contrast to the mock transfectants, only 2 of 75 mice injected with the β4-ΔCYT transfectant subclones had lung metastases. However, the metastatic foci seen in these two mice stained positively with a mAb specific for the human p53 protein indicating that some of the β4-ΔCYT transfectants had the capacity to reach the lungs (Figure 2D). Additional evidence to support the conclusion that α6β1 is essential for the formation of metastases by MDA-MB-435 cells was obtained using the META strain of mice that is highly prone to metastasis formation. The results obtained from injection of these mice were similar to those obtained with the NMRI mice (data not shown).

Although there were very few metastatic foci in the lungs of mice that had been injected with the β4-ΔCYT transfectant subclones, microscopic analysis revealed the presence of mononuclear infiltrates in some of these lungs that were located around small blood vessels (Figure 2B). These infiltrates were comprised primarily of macrophages, lymphocytes, and a few single cells that were apoptotic by morphological criteria and because they stained positively with the ApopTag reagent. Because such infiltrates and apoptotic cells were not observed in the parenchyma of control transfectants with metastases, a reasonable suggestion is that some of the apoptotic cells were β4-ΔCYT transfectants that had

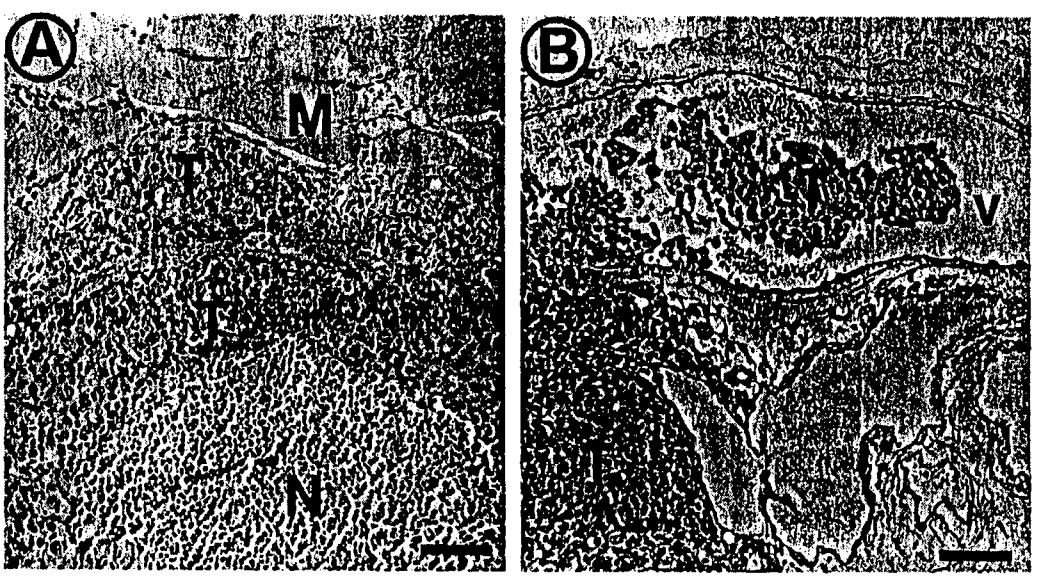

Figure 1. Invasion of the MDA-MB-435 β4-ΔCYT transfectants. A: Paraffin section of a primary tumor in the abdominal region. Tumor cells (T) are present in striated musculature (M) indicating that invasion from the mammary fat pad had occurred. An area of tumor necrosis (N) is also evident. B: Paraffin section of a peritoneal tumor showing tumor cells (T) within a blood vessel (V). Sections were stained with H&E. Scale bar = 90 and 83 μm, respectively.

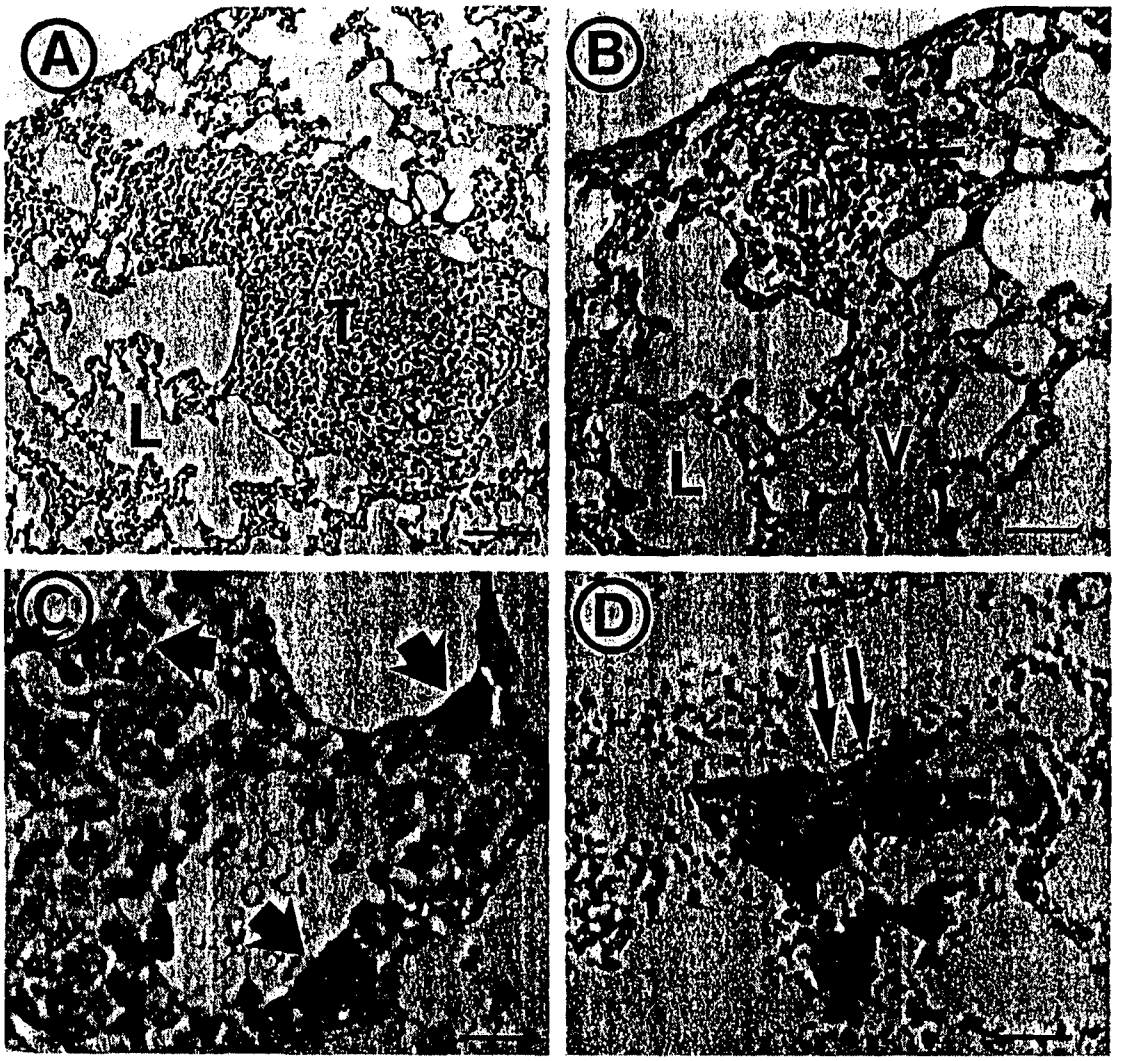

Figure 2. Suppression of lung metastasis by targeted elimination of the α6β1 integrin in MDA-MB-435 breast carcinoma cells. A: Lung metastases are seen in mice injected subcutaneously with the control transfectants but not in the lungs of mice injected with the β4-ΔCYT transfectants (B) with the exception of two mice (Table 2). However, perivascular, granuloma-like infiltrates comprised of macrophages, lymphocytes, and apoptotic cells are evident in some of the lungs of the β4-ΔCYT transfectant-injected mice. The apoptotic cells in these infiltrates were distributed as single cells in areas that were free of necrosis and devoid of neutrophils. These cells also stained positively with ApopTag (C). These infiltrates were seen in 20 of the 75 mice injected with the β4-ΔCYT transfectants. In these 20 mice, a total of 60 such foci were seen. In the control mice, only 3 foci were evident in the 53 mice analyzed. The difference in the number of foci between the control and β4-ΔCYT injected mice is highly statistically significant by the chi-square test ($P < 0.001$). D: Positive immunostaining of tumor cells with a mAb to human p53 in one of the two cases in which lung metasases were seen in the β4-ΔCYT transfectants. This positive staining provides evidence that at least some of the β4-ΔCYT transfectants have the capacity to enter the lung parenchyma. All photomicrographs shown are paraffin sections. A and B are stained with H&E, C is stained with ApoTag with a light hematoxylin counterstaining. T, tumor cells; L, lung parenchyma; V, blood vessel; I, mononuclear inflammatory infiltrate. Arrows indicate an apoptotic cell, arrowheads indicate apoptotic cells that are ApoTag positive, and double arrows indicate p53 immunopositive cells with pleomorphic nuclei. Scale bars = 50, 25, 12.5, and 25 μm, respectively.

spread to the lungs but were unable to survive and establish metastatic colonies.

The observations on the behavior of the MDA-MB-435 transfectants in the lungs raised the possibility that the α6β1 integrin is required for their ability to survive in and colonize distant organs. This possibility was strengthened by examining the behavior of control and β4-ΔCYT transfectant subclones that had been injected directly into the liver.[16] Although the liver is not a major site of spontaneous metastasis for MDA-MB-435 cells (Ref. 15; data not shown), intrahepatic injections provided a direct assay for assessing the ability of the β4-ΔCYT transfectants to grow, survive, and form colonies in a distant organ. Both the control and the β4-ΔCYT transfectant subclones were capable of growth in the liver after intrahepatic injection. However, the control transfectants grew substantially better than the β4-ΔCYT transfectants (Figure 3). Most likely, this difference in growth can be attributed to extensive apoptosis in the β4-ΔCYT transfectants because the tumor masses that arose from these cells had a much higher apoptotic index (3.4 ± 0.41) than did the tumor masses that arose from the control transfectants (0.73 ± 0.14), and they stained positively with the ApopTag reagent (Figure 3). Taken together with the lung results, these data indicate that the inability of the β4-ΔCYT transfectants to develop distant metastases prob-

A.

MDA-MB-435 Transfectants	No. Mice	Extent of Tumor Growth	MI (%) Mitosis	AI (%) Apoptosis	MI/AI Ratio
pcDNA3	10	Heavy (+++)	0.97 ± 0.23	0.73 ± 0.14	1.4
β4-ΔCYT	10	Low (+)	0.95 ± 0.13	3.4 + 0.10	0.28

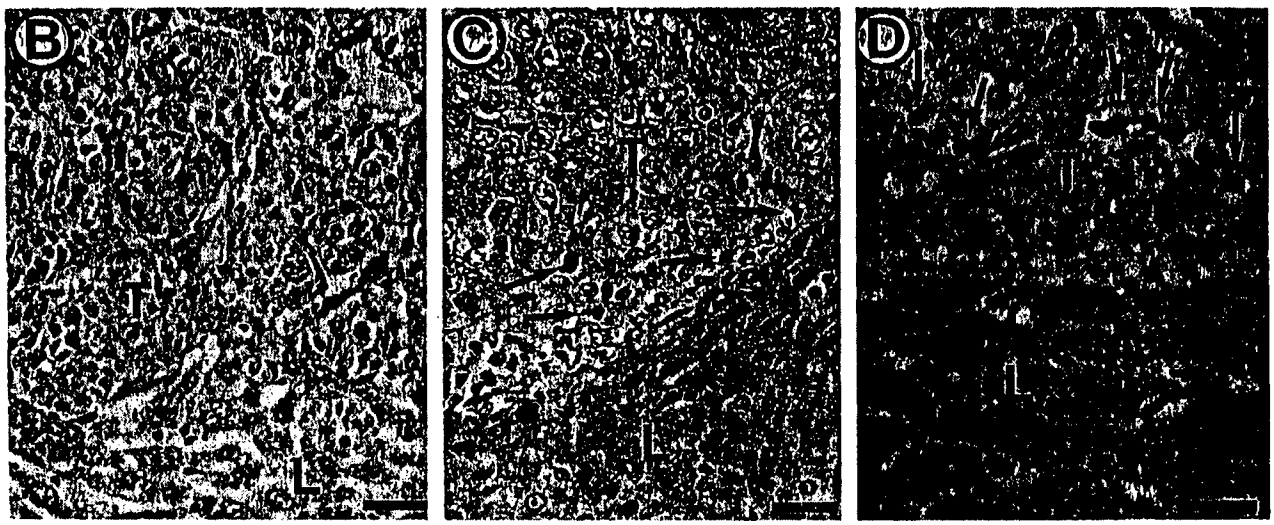

Figure 3. Liver colonization by the MDA-MB-435 transfectants. A: Control transfectants and β4-ΔCYT transfectants were injected directly into the liver parenchyma. Both cell types exhibited tumor growth in the liver, but the size of the tumors was markedly different. Notably, the apoptotic index (AI) of the β4-ΔCYT tumors was significantly higher than that of the control tumors ($P < 0.0002$ *using the Mann-Whitney test*). *The* MI/AI ratio *of the control tumors (1.4) is significantly higher than that of the β4-ΔCYT tumors (0.28)* ($P < 0.03$ *using the Mann-Whitney test*). *The data are presented as mean index (%) and standard error of the mean.* B to D: Morphological analysis of tumor nodules in the liver. B: H&E staining of a paraffin section of a control tumor. C: H&E staining of paraffin section of a β4-ΔCYT tumor. The zone of the tumor closest to the liver parenchyma exhibited numerous tumor cells undergoing apoptosis as evidenced by morphological criteria and by ApopTag staining as shown in D. T is tumor, L is liver. Arrows point to apoptotic cells. B and C, scale bars = 50 μm, and D, scale bar = 25 μm.

ably reflects their inability to survive after they have extravasated into potential metastatic sites.

Discussion

The data presented here argue for an essential role of the integrin α6β1 in breast carcinoma progression. Specifically, we observed that targeted elimination of this integrin in MDA-MB-435 cells reduced the size of primary tumors formed by these cells and diminished evidence of metastatic foci in the lungs of these mice. Both of these effects appear to derive from a decrease in growth and an increase in apoptosis that occurs in the absence of α6β1 expression. Our findings provide insight into the mechanism of breast cancer progression and they also highlight the importance of integrin-mediated events in this disease process. From a clinical perspective, these data provide a functional explanation for the finding that a strong correlation exists between α6 integrin expression in pathological specimens obtained from breast cancer patients and the mortality of these patients.[13]

The technique we used to eliminate α6β1 expression in MDA-MB-435 cells involved expression of a cytoplasmic domain truncation of the β4 integrin subunit (β4-ΔCYT). These cells do not express the α6β4 integrin and expression of this truncated β4 subunit promotes the formation of the α6β4-ΔCYT heterodimer on the cell surface at the expense of α6β1 because α6 associates preferentially with the β4 subunit in comparison to the β1 integrin subunit.[17] Two concerns could be raised by the use of this technique and the interpretation of the data we obtained: 1) expression of β4-ΔCYT could alter expression of other integrins on the cell surface, and the altered expression of these integrins could contribute to the differences in tumor metastasis and survival we observed; and 2) the α6β4-ΔCYT heterodimer could retain signaling properties that affect tumor behavior *in vivo*. We have addressed both of these issues. As stated, the surface expression pattern of other known integrin subunits was

not altered significantly by expression of β4-ΔCYT. This observation was substantiated by the fact that the adhesion of MDA-MB-435 cells to matrix proteins other than laminin-1 was not influenced by expression of β4-ΔCYT. Although we noted variations in α3β1 integrin expression among the MDA-MB-435 subclones used in this study, the levels of α3β1 expression did not correlate with either tumorigenesis or survival of these subclones *in vivo*. With respect to the signaling properties of α6β4-ΔCYT, several observations suggest that it is a nonfunctional receptor. We observed previously that this truncated integrin is unable to stimulate laminin adhesion or invasion through Matrigel, which are two functions we have identified for the α6β4 integrin.[18] Also, ligation of α6β4-ΔCYT failed to induce any detectable increase in tyrosine phosphorylation in contrast to ligation of α6β1 (data not shown).

Our conclusion that the α6β1 integrin is involved in spontaneous metastasis is supported by previous findings that have implicated a role for this integrin in models of experimental metastasis. In these particular studies, the ability of melanoma and fibrosarcoma cells to colonize the lungs after tail vein injection was assessed.[19,20] Using such assays, it was observed that melanoma metastasis could be inhibited by pretreatment of the cells with an α6-specific mAb,[19] and fibrosarcoma metastasis could be blocked using a ribozyme that abrogated expression of the α6 subunit.[20] Although these studies did not address the mechanism by which α6β1 contributes to the metastatic process, they do support the data we obtained. Moreover, other studies have also suggested a link between α6β1 expression and the progression of fibrosarcomas,[21] as well as prostate carcinoma.[22] It will be extremely interesting and informative to determine if α6β1 also functions to promote survival in these cancers.

The involvement of integrins and the signaling pathways they modulate in controlling cell survival has become an explosive area of research.[23–28] The mechanism by which the α6β1 integrin promotes breast carcinoma growth and survival may differ somewhat from recent findings in this field, however, because this integrin does not appear to be essential for the growth and survival of MDA-MB-435 cells *in vitro* even in serum-free conditions. We suggest that MDA-MB-435 cells that express α6β1 can respond *in vivo* to either a specific growth factor, matrix protein, or other cell type to promote their survival. Indeed, a role for α6β1 in promoting survival is supported by the reports that this integrin may be a costimulator of thymocyte[29] and melanoma[30] proliferation. Clearly, understanding how the α6β1 integrin promotes MDA-MB-435 survival *in vivo* and establishing the involvement of specific signaling pathways in this mechanism is an area of investigation that should provide considerable insight into both breast carcinoma progression and integrin signaling.

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